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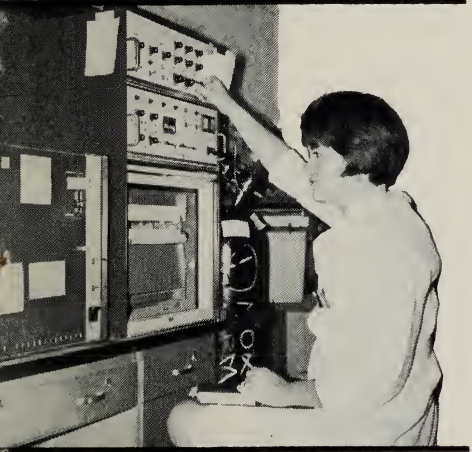

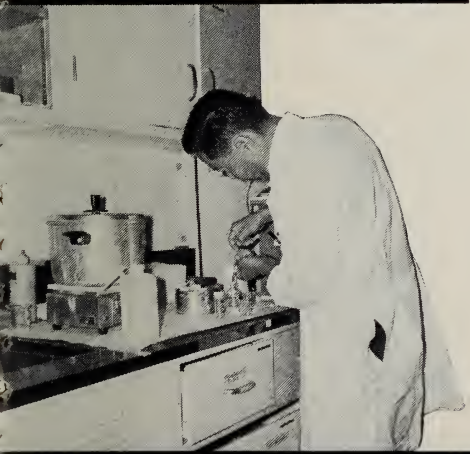

BASIC PROBLEMS OF LIPID METABOLISM OF IMPORTANCE TO MAN

A review of W-44, W-44R, and closely associated publications of the Western Regional Laboratories, 1960–1967

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BASIC PROBLEMS OF LIPID METABOLISM OF IMPORTANCE TO MAN[1]

Ruth Okey, Editor

INTRODUCTION

When revision of Western Regional Project W-44 was planned, most of the laboratories concerned were already engaged in lipid research either with or without additional support beyond regional funds. State projects proposed were evaluated primarily on the basis of their probable contribution to the solution of problems concerned mainly with use of lipids by the human being. Some measurements were to be made with human subjects; others, which demanded data on changes in tissue composition, with laboratory animals.

Research emphasis has shifted during the five years covered by W-44 R. We have become increasingly conscious that the work done in our regional laboratories under W-44 must be considered as only a small fraction of a worldwide effort toward understanding the factors which may affect the physiology and pathology of lipid metabolism.

This review covers 46 papers either published with W-44 and W-44 R accreditation or based on work directed by the members of the W-44 technical committee and dealing with problems very closely related to W-44 R but not necessarily directly supported by regional funds. The studies may be classified roughly as those dealing with 1) analytical methods and their modification for specific uses; 2) effects of diet, age, sex, exercise, and environment on serum lipids and cholesterol, primarily in human subjects; and (3) attempts to determine the intermediary reactions involved in changes in lipid composition of blood and tissue, and how such changes may be affected by endocrine or dietary influences. The latter studies involved the use of laboratory animals and their sacrifice for tissue analyses. Publications during the period 1960 to 1967 are included. Consequently some of the reports cover work actually supported by W-44 but not published before the summary of that project was published (*12*).

The problems of the editor have also been complicated by differences in cost accounting in the various laboratories. Some have kept separate accounts for regional funds and have acknowledged regional support only for projects largely or completely financed by W-44 R funds, while others have put together all available funds for lipid research and acknowledged partial support from several sources. In the interests of continuity, it has been necessary to include fairly comprehensive reviews of relevant papers, by members of the technical committee, which do not acknowledge W-44[2] support. In general, regional funds have covered only a relatively small fraction of the cost of the Western Regional research reported.

It is obvious that a project such as W-44 R cannot be adequately reported within too-fixed an outline. Objectives, procedures, and conclusions are plural rather than singular. Very little of the work reported would have been possible without support in addition to that furnished by W-44 R funds, i.e., state funds and those from the National Institutes of Health and various foundations. Results do indicate, however, that regional support has been justified on the following grounds: It has made possible 1) cooperative work on new methods as well as comparison of analytical results when the same blood and tissue samples were handled by procedures in use in different laboratories; 2) discussion by the whole group of representatives at the annual meeting of the technical committee, effecting better planning, more critical evaluation of results, and presentation of many new ideas; 3) workshops for less formal training of personnel from one laboratory in methods developed in another; 4) seminars and symposia at the annual meetings of the technical committee, which have contributed to understanding the over-all problems of fat and lipid metabolism. In general, the objective of regional research under W-44 R has been directed toward providing the individual laboratories with many of the advantages of membership in the larger and better staffed groups, without interfering with participants' freedom to work on the problems dictated by interests, talents, and available facilities in each state.

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[1] Submitted for publication March 11, 1968.

[2] In this and some subsequent cases W-44 is used to refer to the original W-44 and the revised W-44 R projects as one entity.

METHODS EMPLOYED IN LIPID RESEARCH

While methodology is usually of small interest to readers not directly involved in a research project, nevertheless the period covered by W-44 and W-44 R has seen such spectacular development in techniques for lipid separation and analysis that some mention of methods seems mandatory. Furthermore, the laboratories participating in W-44 have made considerable contributions in the area of lipid methodology. Since 1950, we have learned to expect precise data on lipid composition in a matter of hours, and with samples measured in milligrams and micrograms. Before that time, to achieve such results required large samples, and weeks or even months of work with such painfully slow techniques as combustion, molecular distillation, fractional crystallization, and so forth.

Lipid Analysis

Preparation of blood and tissue samples for analysis has been greatly improved during the periods of W-44 and W-44 R investigations. Quick-freezing of tissue samples with dry ice, the use of refrigerated centrifuges, and vacuum rotary evaporators are only a few of the improved procedures which have made lipid analysis less tedious as well as more accurate. The demonstration that lipids in freeze-dried or lyophilized tissues remained stable for considerable periods of time (*1*) added greatly to the possibilities for accurate work in laboratories where technical assistance is limited. The advantages and disadvantages of different procedures for extracting specific lipids from blood and tissues have been discussed in the methods sections of various W-44 papers. An exchange of information on experiences with different techniques has been an especially useful function of technical committee meetings.

Micro adaptations of two plasma cholesterol methods (*2*) and (*3*) have been extensively used in studies with human subjects.

An ingenious method for separation of white cells and platelets and determination of cholesterol in blood fractions has been developed at Oregon (see page 7).

An evaluation of the analytical procedures for determination of cholesterol in the various laboratories participating in the W-44 study was also carried out by Hawthorne's group (*4*). Commercially lyophilized plasma samples sent out from the Oregon laboratory were analyzed in the different states by the methods regularly used. The considerable variation in results emphasizes the necessity for standardization of procedures for use with a given problem. For example, a procedure satisfactory for use clinically with human serum (range 120 to 300 mg/100 ml) is not necessarily the best for rat plasma (range 30 to 70 mg/100 ml); and one that may be satisfactory for a chromatographically separated cholesterol ester fraction may give poor results when applied to an unfractionated plasma.

The increasing clinical emphasis on the part played by polyunsaturated fatty acids in the metabolism of cholesterol and in the etiology of atherosclerosis has led to development of better methods for determination of the fatty-acid constituents of blood and tissue lipids. Separation by column chromatography has, in general, followed the method devised by Fillerup and Mead (*Proc. Soc. Exp. Biol. Med.* **83:** 574 [1953]). A lipid extract in an inert solvent, such as petroleum ether, is placed on a column packed with specially prepared silicic acid or other absorbent material. The extract is successively eluted with various solvent mixtures. The usual fractions separated are: 1) cholesterol ester; 2) triglyceride; 3) free fatty acid and free cholesterol; 4) phospholipid. An adaptation of this procedure, developed at California (*5*) for use with small samples, has been widely used, especially for animal experiments in which only small amounts of lipid material were available.

Characterization of the fatty acids in each lipid fraction became possible with the development of gas-liquid chromatography. Fatty acids from the column fractions are methylated and are, in essence, fractionally distilled on a column packed with an inert material, such as fire clay, which is coated with another inert material, a liquid at the temperature at which the methyl esters vaporize. After the individual esters pass off the column, their presence is recorded and their identification and proportion are measured by comparison with a mixture of methyl esters of known fatty acids. The W-44 contributions to this technique have been: 1) a method for translating percentages of the individual fatty acids to actual amounts by the use of a measured quantity of C^{17} fatty acid methyl ester as an internal standard (*6*); 2) a simplified procedure for locating the position of the double bonds in the polyunsaturated fatty acids that occur in nature (*7*). Both of these procedures have been of value in the quantitation and characterization of a variety of tissue lipids.

A third technique which has recently been applied to separation of lipids is thin-layer chromatography. In this procedure, glass or plastic sheets are coated with specially prepared silicic acid. Small samples of the lipid extracts are "spotted" at or along one edge of the plate. The different constituents of the extract can be made to move up the plate at different rates by "development" in different solvent mixtures, and can thus be separated into distinct spots. Known standards can be applied to the plates at the same time, which makes for better controls. The procedure is relatively easy and inexpensive, and allows good resolution of many lipid constituents heretofore separated only with difficulty. At present, the Oregon and California laboratories are making extensive use of this technique for separation of individual phospholipids (see pages 7 and 21–22).

Animal-feeding Experiments

Discussion of methodology would not be complete without some mention of our observations on the effect of fasting, meal feeding, pair feeding and other variations in time of access to food on the lipid content of blood and tissues. An early report (Lyon, Masri, and Chaikoff, *Journal of Biological Chemistry* **25:** 196 [1952]) stated that the capacity for synthesis of cholesterol from C^{14}-labeled acetate was largely lost in liver slices from fasting animals. Another observation (Cockburn and Van Bruggen, *Journal of Biological Chemistry* **234:** 481 [1959]) showed that, in rats, refeeding after a fast resulted in an accelerated rate of synthesis of cholesterol and fatty acid *in vivo*, lasting for several hours. Both studies emphasized the importance of control of the interval between the last feeding and autopsy if the values for tissue lipids were to be meaningful. It seemed reasonable to expect, moreover, that composition of the diet might influence the extent to which time of withdrawal of food preceding autopsy might, in turn, influence blood and tissue lipid values. The problem of securing uniform time intervals between feeding and autopsy is difficult with animals that have irregular eating habits, such as rats.

Several studies in the Western Regional laboratories have dealt with various aspects of this problem. Mayfield and her associates at Montana (*8*) used diets containing high levels (57.8 to 62 per cent) of nonfat milk solids with about 35 per cent of fat given, respectively, as corn oil, margarine, or butter. They measured differences in serum and liver lipids in animals either fed to time of autopsy or fasted for 10 to 12 hours preceding sacrifice. Liver weights decreased sharply in fasting animals (males, mean decrease 1.5 gm; females, mean decrease 1.22 gm). While percentages of total liver lipids and cholesterol (as percentage moist weight) increased in the females less consistently than in the males, serum cholesterol decreased with fasting as did serum phospholipid:cholesterol ratios. In nonfasted animals, livers of females had lower percentages of cholesterol than did those of males. Serum lipid differences between rats fed animal fat (butter) and vegetable fat (corn oil or margarine) were significant only for the nonfasted group. These results confirmed earlier findings of Okey *et al.* (*9*) concerning the effects of overnight fasting preceding autopsy. A large variation in eating times noted in individual rats fed *ad libitum* indicated definite advantages for use of a fasting period followed by feeding four to six hours before autopsy, in order to secure uniformity of effects of food intake on lipid synthesis from C^{14}-labeled acetates (*9*). Overnight fasting can be considered to lessen differences due to immediate absorption and temporary retention of nutrients in the liver. Except for meal-fed animals killed at uniform intervals after a meal, the mean differences between groups have been found to be more consistent in rats subjected to an overnight fast. The fasted animals do, however, reflect relatively permanent changes in tissue composition resulting from type of diet rather than short-time changes resulting from the ingestion of food.

Animals fed unbalanced or deficient diets usually eat less than do controls fed adequate diets. In order to match weight gain it is sometimes necessary to employ a paired-feeding technique—that is, to feed control rats only the amount of the control diet equivalent to that eaten by the animal on the unbalanced or deficient diet. These pair-fed controls, which are extremely hungry, will usually consume the food offered within a short time and will, therefore, qualify as being meal-fed. Similarly, the "trained feeding" regimen in which animals have access to food for only a limited period or periods per day is likely to result in changes in liver and serum lipid concentration and, indirectly, to affect the concentration of fat and cholesterol in serum and tissues. Such differences are incidental to changes in size of stomach and to time during which food is present in and absorbed from the alimentary tract. The above adaptations take place as the animal adjusts itself to any plan of feeding (*10*).

A number of measurements concerned with effect of time of access to food have been made in the Western Regional laboratories, most of them as controls for procedures used in investigating the effect of feeding individual rats. One of the more interesting findings was a sex difference in the effect of restriction of time of access to food on plasma and liver lipids, which is noted under the discussion of sex differences in lipid metabolism (see page 17).

STUDIES WITH HUMAN SUBJECTS

Serum Cholesterol: Age and Diet

Measurement of serum cholesterol in human subjects of different ages was begun as part of the original W-4 project, and continued under W-44. Some of the publications completed after the beginning of W-44 R present work actually done with W-4 funds. Summary reports of the W-4 work on cholesterol in human subjects (*11*) and of later work under W-44 (*12*) have been published as bulletins. Measurements showed some increase in human serum cholesterol with age. In women, a fairly sharp increase occurred at about age 55, and lasted until about 75. No particular increase occurred in men at age 55, but there was a sharp drop after 75. In California a follow-up was made of the original measurements on a group of 50 men who lived in a county home. These men had very low serum cholesterols with very low fat intakes as well as low intakes in vitamin A, carotene, and ascorbic acid. The mortality rate in these county-home men in the eight years following the W-4 study was nearly three times that of the men living at home. The mortality rate of women living in their own homes was only about half the rate of the men living in their own homes. These data, as Dr. Morgan points out *(11, 12),* contradict somewhat the generally accepted concept that serum cholesterol varies with proneness to development of atherosclerosis. Another study, likewise done under the auspices of W-4 and W-44 (*13*), gave higher values for serum cholesterol in women living at high altitudes than did the data from California.

A series of studies of adolescents and preadolescents, also W-4-supported, was reported in the period covered by W-44 (*14*). While the cholesterol intakes of boys were higher than those of the girls, they showed no correlation with weight. Serum values for girls were higher than those for boys. There was some positive correlation between ascorbic acid, vitamin A, and cholesterol. In Montana (*15*), in two counties with variable amounts of sunshine, there was some correlation between exposure to sunshine and serum cholesterol. This was also true for older women in Wyoming (*11*). Both reports indicate a relationship of altitude to serum cholesterol.

A seven-year study correlating physical characteristics in preadolescents and adolescents with serum cholesterol was made at Utah (*16*). This study dealt with 152 girls and 169 boys and was made in two counties. Serum cholesterol values were taken at two-year intervals, and were correlated with measurements of hemoglobin, body weight, and build. Fingertip blood was used, and the determinations were carried out by the modified Sperry-Webb procedure of Wilcox and Galloway (*2*). Serum cholesterols did not vary significantly between ages 7 and 17, except possibly for some high values at adolescence. Siblings in 62 families were compared. Girls had significantly higher serum cholesterol levels (51.6 mg/100 ml free; 187 mg total) than did boys (48.9 mg/100 ml free; 178 mg total). A slight elevation of serum cholesterol was observed in persistently overweight girls, whereas no change was observed in boys of different body builds. The data did not show the paralled relationship between hemoglobin and cholesterol previously reported from Montana (*14*). Certain family patterns for serum cholesterol were noted. The brothers and sisters of subjects with serum cholesterol levels of 200 mg/100 ml or over had higher values (32 mg or more) than did the siblings of subjects with cholesterol levels between 185 mg and 200 mg/100 ml.

Hawthorne and others in Oregon (*17*) evaluated serum, red, and white cell fractions in the blood of 18 women and 14 men, ages 22 to 91 years. The ranges for serum for women were 122 to 289 mg/100 ml, for men 121 to 338 mg. Red blood cell (RBC) values for women were 90 to 141 mg/100 ml; for men, 82 to 151. White blood cell (WBC) values were 536 to 2,214 mg/100 ml of cells for women, 932 to 2,794 mg for men, except for one very high value, 5,124 mg, for a sample taken a day after a coronary occlusion. Ratios of free to total cholesterol in serum were 0.25 to 0.33 for women, 0.23 to 0.39 in the men. The cholesterol in red blood cells was almost all free; in white cells, 30 to 81 per cent free for women, 23 to 96 per cent for men. Values tended to be higher in late middle age. Because of new methods used, and differences in age and condition of subjects, statistical evaluation was made only for replicate analyses. This paper is noteworthy for the high values reported for white-cell cholesterol and the possible implication that cholesterol may be important in the metabolic function of these cells.

A later paper from Oregon (*18*) reports thin-layer chromatography (TLC) fractionation of the phospholipid classes in human blood constituents. Phospholipid constituted about two thirds of the RBC lipid, 40 to 70 per cent of that in WBC and 65 to 80 per cent of that in platelets.

Approximately two thirds of the serum phospholipid was in the phosphatidylcholine (PC) and phosphatidyl serine (PS) fractions. There was little or no phosphatidyl ethanolamine (PE). Red cell PL contained more sphingomyelin (SPH) and PE (about one fourth of total PL) and less PC and PS (about two fifths of PL). Platelets and WBC PL were much alike. PC and PS made up a little over one half of the PL; SPH and PE about one fifth.

Comparison of the Oregon findings with human subjects with a California paper on distribution of fatty acids in the serum and RBC of rats (see p. 18) points to the desirability of investigating the fatty-acid composition of the lipid fractions in human blood constituents in relation to susceptibility to atherosclerosis.

Environmental Temperature and Exercise

Thompson *et al.* in Arizona (*19*, *20*) followed a preliminary study of the effects of environmental temperature on basal metabolic rate and serum ascorbic acid with a study of the effects of environmental temperature and exercise on basal metabolic rate, protein-bound iodine (PBI), and total cholesterol. Seven student nurses and 11 postmen, all healthy, served as subjects. Measurements were made during December through February (minimum temperature, 38° F) and during June through August (maximum temperature 102° F). The mean age of the nurses was 19.6 years, of the postmen, 39.7 years. Dietary records were kept and calculations made, but the diets were not controlled. The nurses worked in ventilated, but not air-conditioned rooms. Postmen were outdoors during the winter and summer months. In both cases there was sustained moderate muscular exercise. The men showed a high PBI in winter—7.3μg against 4.9μg per 100 ml. The women had no great variation with season. Basal metabolic rate for men was 7.3 per cent below the Mayo Foundation standard in summer and 3.6 per cent below it in winter. That for women was 8.2 per cent below the Mayo standard in summer and 3.5 per cent in winter.

Women had no seasonal change in serum cholesterol, but low values were commonly observed near the time of menstruation. Three women were observed daily. Cholesterol values for men were 9 per cent lower in summer (mean values, 233 vs. 255 mg per 100 ml). The study concluded that changes in PBI and cholesterol in men were probably associated with hemo concentration resulting from a larger consumption of cold

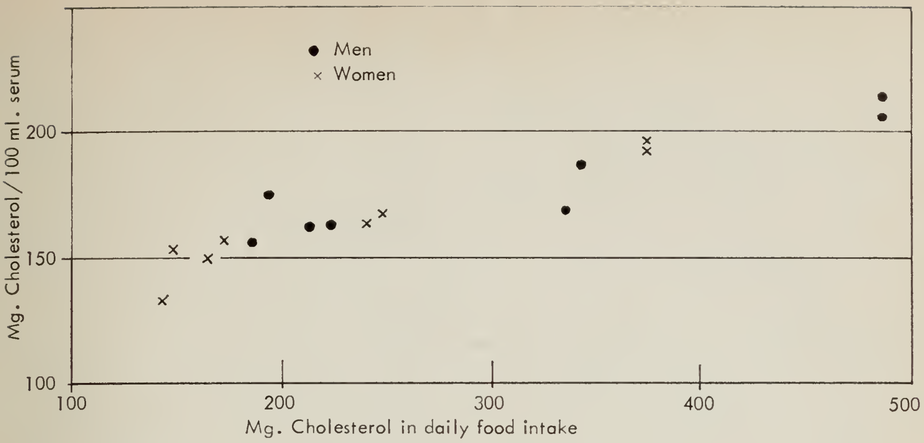

Fig. 1. Relationship between serum cholesterol values and dietary cholesterol. (From: Wilcox, E. B., and L. S. Galloway, Jour. Amer. Diet. Assn. **38:** 229 [1961].)

drinks. The menstrual changes in women confirm the early findings of Okey and Boyden (*Jour. of Biol. Chem.* **72:** 261 [1927]). Similar changes have been observed elsewhere.

Effect of Dietary Fats

Studies at Utah on young human subjects included observations of serum cholesterol in subjects fed different dietary fats (*21*). Measurements of serum cholesterol were made three times a week by the Wilcox and Galloway modification of the Sperry-Webb method. Seven food fats were tested—butter, margarine, hydrogenated coconut oil, coconut oil, lard, hydrogenated lard, and corn oil. Subjects were in two diet squads, each consisting of four men (mean age 24) and four women (mean age 21.4). Test periods were 15 days. An adjustment period, in which 35 per cent of the calories was furnished by fat of mixed origin, preceded the experimental period. Caloric intakes were adjusted individually to maintain weight, and varied from 2,100 to 2,300 per day for women, 2,900 to 3,100 for men. Seventy-eight per cent of the fat intake was considered to have been furnished by the fats under investigation (fig. 1).

Serum cholesterol was lowered on the test diets except when butter was fed. Differences from the control period were significant for margarine, cottonseed oil, hydrogenated cottonseed oil, and corn oil. Others were not great enough to be statistically significant. The differences between the values for the butter and lard periods were not significant, but highly significant differences were observed for the mean for the two periods during which only corn oil was fed, and either period when only butter was fed (fig. 2). There was good correlation between cholesterol intake and serum cholesterol. The data showed some holdover of low values from the corn oil period. This agreed with findings of a previous study at California (*22*), with a group of 30 prison volunteers (men, mean age 50) fed safflower oil for one month. In the latter study, one month on the regular prison diet, rich in lard, was followed by a month on the experimental diet, containing safflower oil. There was then a month during which subjects ate the regular prison diet, followed by a month with coconut oil. Total plasma lipid, free and total cholesterol and lipid phosphorus, and plasma fatty acids were fractionated. Proportions of fat calories were very much alike in the Utah and California studies, but the day-to-day uni-

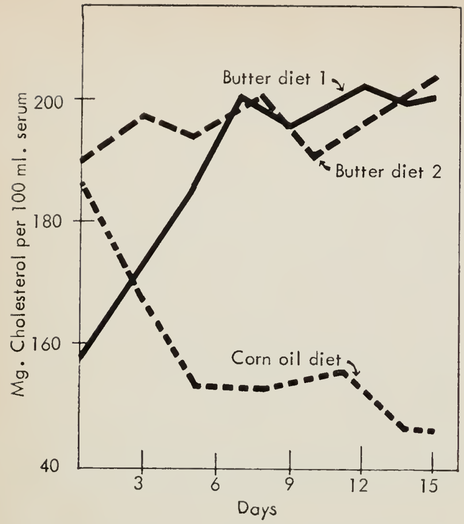

Fig. 2. Differences between serum cholesterol levels for two diet squads, each fed butter or corn oil. Corn oil graph represents the mean for two squads that reacted similarly. Data for butter squads were charted separately. (From: Wilcox, E. B., and L. S. Galloway, Jour. Amer. Diet. Assn. **38:** 27 [1961].)

formity of intake was much more closely controlled in the shorter Utah study, where subjects were fed in the laboratory. In the California study the men passed through a cafeteria line; food taken and plate wastes were recorded. The relatively high serum cholesterols observed on the butter and lard diets can be taken as evidence that animal fat tends to produce higher serum cholesterols than do vegetable fats—especially vegetable fats that contain no cholesterol and are rich in linoleic acid. The California study showed a high correlation between linoleic acid intake and plasma linoleic acid.

Another study by Wilcox *et al.* (*23*) at Utah measured the effect of milk intake with concomitant variations in fat and protein intake, and of exercise, on serum lipids of a group of 24 university athletes who were taking part in competitive sports. Nine diets were fed in the laboratory. Milk intakes were: zero, 1 quart, or 2 quarts daily, each level fed as part of diets furnishing 10, 13.4, and 16.8 per cent, respectively, of the calorie intake. Calories were adjusted to the needs of the individual. Eight subjects were tested on each diet. The subjects were either in training for, or actually participating in football, basketball, or track. Serum lipids were determined at the beginning of the investigation, and before and after a measured amount of additional exercise in the form of athletic performances. Laboratory measurements included serum cholesterol, total lipids, and lipid phosphorus. None of these was affected by the changes in milk intake or the protein intake. The amount of additional exercise for the experiment had no significant effect in the trained subjects.

Mean dietary cholesterol intakes varied from 959 to 1,233 mg per day. The lowest intake occurred in subjects receiving diets providing only 10 per cent of the total calories as protein; the highest was in subjects on the no-milk diet. Fecal nitrogen excretion was not influenced by milk or protein intake. Urinary excretion of nitrogen was similar for all subjects on the milk diets, and increased as the level of dietary protein increased. Added exercise caused a slight decrease in urinary nitrogen with all the diets.

Dietary cholesterol intakes were more than double those of the nonathletic university men who participated in a previous study (range of means varied from 187 to 486 mg). Serum cholesterol values of the athletes were within the range of values obtained in the study that did not include exercise, with the possible exception of the subjects in the previous study (*21*) who had received 187 mg dietary cholesterol. The benefit of regular exercise in controlling cholesterol levels is indicated rather than fully proved.

These studies from Utah are of particular importance because of the care with which dietary intakes were controlled, and the careful measurement of activities. It is interesting that both the Utah and

California studies indicate that the effect of composition of a dietary fat on serum lipids in human subjects may last over a considerable period of time. While the Utah studies were carried out for shorter periods than that of the prison study in California, it is obvious that in both cases the time was not great enough to eliminate entirely the effect of the diet prior to the experimental period. Altogether, the studies with human subjects represent a very substantial contribution in a field in which experimental control is hard to achieve.

STUDIES WITH LABORATORY ANIMALS

Studies with human subjects are necessarily limited to those that can be made without detriment to health. The scope of such studies is further limited by expense and by difficulties in control of such factors as nutrient intake, energy output, and the many genetic and environtal factors that influence metabolism. Supplementation of human studies with measurements made with laboratory animals has contributed much of our information with regard to changes in tissue composition, enzyme activity, and the effect of hormones on lipid metabolism. With due regard to species differences in metabolic processes, we recognize that the best hope for solution of many metabolic problems lies in animal experimentation.

The original plan for the animal experiments to be made with the assistance of W-44 funds was to study the effect of variation in the supply of different essential nutrients on lipid metabolism. As the work progressed, it has inevitably been concentrated in areas where findings have been especially interesting or have been closely related to other problems under study by the laboratory personnel. For example, studies on the effect of protein intake have led to intensive study of the use of methionine as a source of methyl in the formation of lecithin. Studies on essential fatty acids have been related to problems of conservation, transport, and use of linoleic and arachidonic acid in the esterification of cholesterol and the function of phospholipids. Observations of sex differences in tissue lipids of rats have led to studies on the effect of estrogens and androgens on lipid metabolism in castrate animals (California). Studies on effect of altitude and iron intake have led to measurements of succinic dehydrogenase in relation to cholesterol metabolism of rats (Montana).

Effect of Fat Intake on Lipid and Cholesterol Metabolism

Data in a series of reports from California, published between 1955 and 1960, furnished background for W-44 investigations dealing with the effect of different dietary fats on the plasma and tissue lipids of rats. These were seven-week studies with groups of 10 male and 10 female rats, each placed, at weaning, on synthetic diets adequate for normal growth. The distinctive characteristics of these diets were: protein made up of 10 per cent egg albumin and 5 per cent casein; enough added biotin to compensate for the avidin; and choline kept at 750 mg/kg diet, i.e., about the minimal level adequate for growth when given with a protein high in methionine. This diet has been used in a number of subsequent studies, both at California and elsewhere.

The first paper (*24*) dealt with the comparative effects of cottonseed and coconut oils at 5, 10, and 15 per cent of the diet. Results were responsible for the choice of a 10 per cent level of fat in a comparison of effects of 13 fats of widely different composition (*25*). Data for serum cholesterol are shown in table 1 and for liver lipids and cholesterol in figures 3 and 4. They posed a number of

TABLE 1*
SERUM CHOLESTEROL OF RATS FED THE INDICATED FATS AS 10 PER CENT OF A SYNTHETIC DIET

Fat in diet	Approx. dienoic acid in fat	Iod. no.	Serum cholesterol levels in: Rats fed no cholesterol		Rats fed 1 per cent cholesterol	
			Males	Females	Males	Females
	per cent		*mg./100 ml*	*mg./100 ml*	*mg./100 ml*	*mg./100 ml*
Menhaden oil†	6	143	53.5 ± 3.2	50.0 ± 2.7	68.1 ± 7.0	81.6 ± 10.7
Safflower oil	75	137	66.0 ± 5.3	66.9 ± 2.8	71.9 ± 4.3	219.0 ± 39.9
Corn oil	52	113	72.0 ± 4.9	62.6 ± 2.9	54.9 ± 3.0	69.2 ± 3.6
Soy oil	52	112	63.5 ± 5.0	67.9 ± 5.2	60.2 ± 3.7	95.0 ± 15.0
Cottonseed oil						
Straight	54	104	67.2 ± 4.0	76.9 ± 3.8	62.5 ± 4.6	158.9 ± 28.0
Hardened‡	49	97	69.5 ± 3.6	69.5 ± 3.6	54.7 ± 3.4	86.0 ± 12.0
Peanut oil	25	83	74.0 ± 4.7	74.0 ± 3.0	65.4 ± 5.5	68.5 ± 5.5
Olive oil	7	82	83.9 ± 4.0	75.1 ± 2.7	71.0 ± 5.6	197.0 ± 27.6
Lard	12	67	62.7 ± 3.2	70.8 ± 4.0	69.0 ± 3.6	133.0 ± 18.2
Shortening§	1 to 4	62	69.2 ± 2.8	74.1 ± 4.0	72.3 ± 6.8	164.0 ± 22.0
Butter oil	3	35	59.8 ± 5.9	58.1 ± 2.8	61.3 ± 3.3	71.2 ± 6.5
Butterfat	3 to 4	26	68.2 ± 4.5	68.1 ± 3.0	71.5 ± 3.8	81.3 ± 4.0
Coconut oil	1 to 3	11	72.4 ± 4.8	85.8 ± 3.4	96.2 ± 8.0	129.0 ± 15.0

* Table adapted from Okey *et al.*, *Metabolism* **8**:241 (1959).

† Dienoic acid chiefly linoleic ($C_{18}H_{34}O_2$); menhaden oil contained approximately 37 per cent polyenoic acids, possibly branched-chain acids of more than 18 carbons.

‡ Oil consisted of 10 per cent completely hydrogenated and 90 per cent unchanged oil; typical of some margarine formulas.

§ Cake shortening containing some hydrogenated fat and emulsifying agents (0.71 per cent lecithin).

questions which later investigations have attempted to answer as better analytical procedures were developed: 1) Why was there greater storage of liver triglyceride and cholesterol ester in the livers of male than of female rats, especially when cholesterol was fed with a dietary fat rich in long-chain fatty acids? 2) Why did two-thirds to three-fourths of the female rats in any one group tend to have high serum cholesterol levels while the others had values that ranged around the mean for males? 3) To what extent did the use of a protein rich in methionine with a diet containing a marginal to "low adequate" amount of choline exaggerate sex differences?

At the time these studies were made, gas-liquid and thin-layer chromatographic techniques were not available. Procedures included column separations of liver lipids into: 1) cholesteryl ester; 2) triglyceride; 3) free fatty acid, mono-diglyceride, and free cholesterol; 4) phospholipid fractions. Proportions of saturated, mono-, di- and polyenoic acids were estimated spectrophotometrically after alkali conjugation.

Wilcox and Galloway (*26*) at Utah made a somewhat similar five- and eight-week study of rats fed lard or cottonseed oil with and without cholesterol. The basal diet differed from that used at California in that it contained 20 per cent casein and 0.1 per cent choline—i.e., the choline-to-methionine ratio was higher. Fats were fed at the 5, 13.5, and 20 per cent levels, and the calorie intake was adjusted to that consumed by the 5 per cent group. Protein calories were kept constant. Data in this study also showed that males had larger livers and higher total liver lipids than did females. Serum cholesterol values and serum total lipids were somewhat higher for the lard than the cottonseed oil groups. Most of the increase in liver cholesterol in the cholesterol-fed groups was in the esterified fraction. A small group of females fed cholesterol with 20 per cent lard had con-

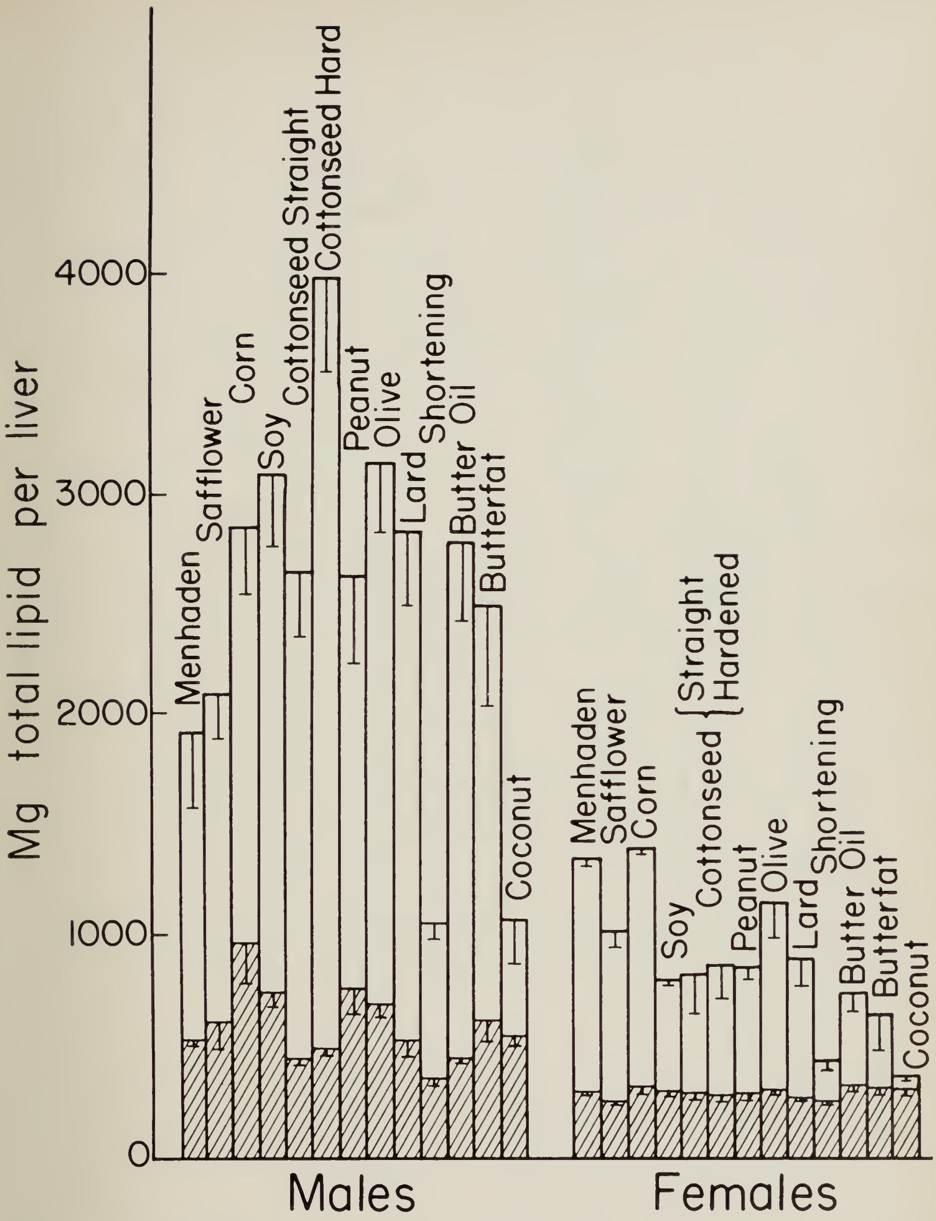

Fig. 3. Total liver lipids of rats fed various dietary fats. Heights of shaded portions of columns represent means for rats fed no cholesterol; total heights of columns, means for rats fed the same diets with 1 per cent added cholesterol. One standard error is indicated by each dropped line. (From: Okey, R., et al., Metabolism **8:** 241 [1959].)

sistently high (111 and 163) serum total cholesterols. These results agree with the California findings. Alfin-Slater and others (*Jour. Nutrition* **62:** 273 [1957] and **65:** 469 [1958]) had also found higher liver cholesterol values for male rats fed lard with cholesterol than for those fed cottonseed oil and cholesterol. These experiments were continued for periods of time longer than those of Wilcox and Galloway (*26*), however.

The Utah paper also reported results for lecithin fed at 5, 13, and 20 per cent levels. (The investigation was particularly interesting in view of Adelle Davis's recommendations for a high lecithin intake in *Let's Get Well,* Harcourt Brace, 1965.) With 20 per cent lecithin, the Utah rats showed diarrhea and a marked decrease in weight gain. When the two higher levels of lecithin were fed instead of fat, serum and liver cholesterol remained within normal ranges even when 1 per cent cholesterol was added to the diet. Five per cent lecithin with 8 per cent lard had about the same effect as 5 per cent lecithin alone; that is, there was some lowering of liver cholesterol. The liver values for animals fed cholesterol with lecithin and lard were about equal to those for animals fed the same amount of lard without cholesterol or lecithin. It is difficult to evaluate the significance of the figures because variability within groups is not given.

The Utah report also included results of some studies on the effect of alfalfa saponin[3] in a group of rats fed 20 per cent lard. The saponin decreased weight gain to a greater extent when fed with cholesterol than when fed alone. The cholesterol-fed rats given saponin had lower serum cholesterols than did those fed the same levels of fat and cholesterol without the saponin. The saponin apparently formed an insoluble or at least nonabsorbable compound with cholesterol in the intestinal tract.

Another study in California (*27*) reported the differences in composition of plasma lipids from the hepatic and femoral veins of female rats fed 10 per cent safflower oil with and without cholesterol. Plasma from the hepatic veins always had the higher percentage of cholesteryl ester, indicating that the liver was removing cholesterol from the blood. This was especially interesting since the level of arachidonic acid in plasma cholesteryl ester is much higher than that in liver cholesteryl ester. Most of the arachidonic acid in liver is to be found in the phospholipid. The question is whether the liver is better able to transform ester cholesterol to bile acid when cholesterol is combined with arachidonic acid (as it is in plasma) or whether the arachidonic acid from plasma cholesteryl ester is being used as a source of arachidonic acid in the formation of liver phospholipids. In view of the postulate by Swell *et al.* (*Proc. Soc. Exp. Biol. Med.* **104:** 325 [1960]) that a high level of serum arachidonic acid can be taken as an indication of the resistance to atherosclerosis in a number of species of animals, and the fact that the rat is among the most resistant animals known, further investigations of hepatic effect on plasma lipids may be very interesting—particularly so when techniques for analysis of small samples of plasma are perfected.

The length of time after withdrawal of a diet high in linoleic acid that human subjects maintained an increased linoleic acid content of serum, and experimental animals maintained a relatively high content of linoleic acid in liver and serum, suggested that adipose tissue might store a reserve supply of linoleate. Adipose tissue content of fatty acids after transfer of rats from a fat high in linoleate to one containing little linoleate was therefore investigated (*28*). Experiments were also conducted on liver and

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[3] See Peterson's review in the *Amer. Jour. Clin. Nutr.* 6: 644 (1958), for discussion of the whole question of the influence of saponins on cholesterol and plant sterol absorption.

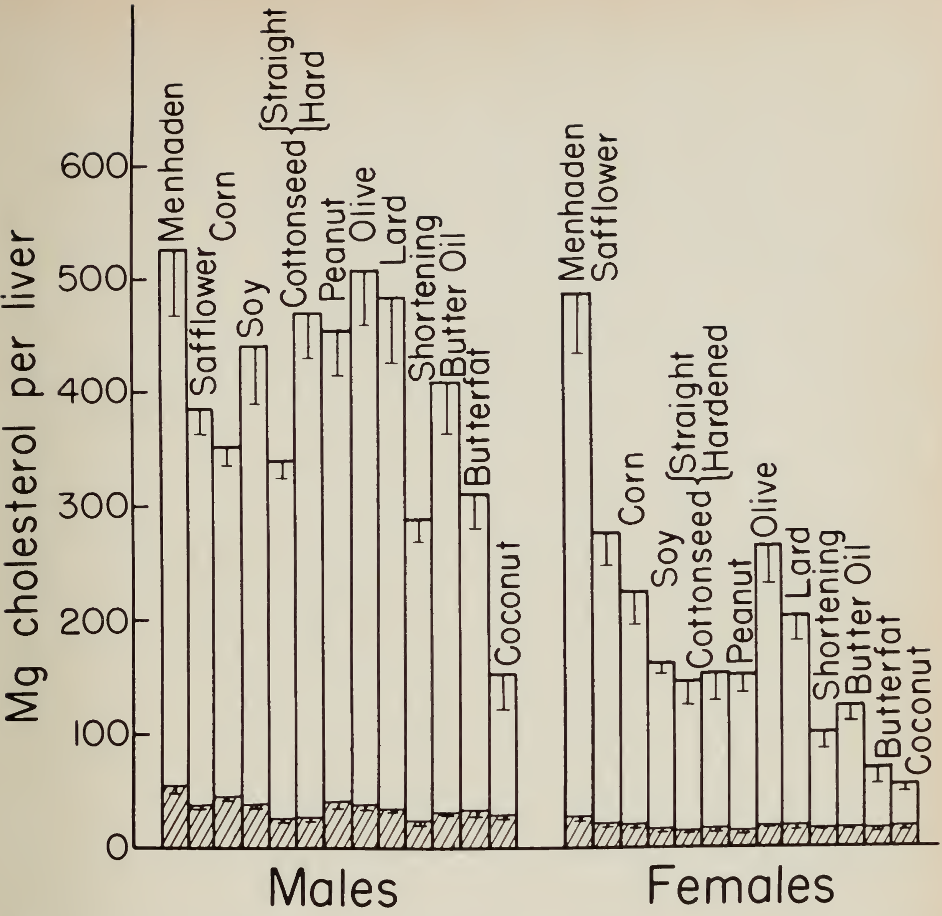

Fig. 4. Total liver cholesterol of rats fed various dietary fats. Heights of shaded portions of columns represent means for rats fed the same diets with 1 per cent added cholesterol. One standard error is indicated by each dropped line. (From: Okey, R., et al., Metabolism **8:** 241 [1959].)

plasma lipids in the same animals (*29*). Safflower oil (76 per cent linoleate) and coconut oil (3 per cent linoleate and about 55 per cent laurate) were chosen for contrast. Rats were fed coconut oil diets (10 per cent fat) for three weeks. They were then divided into three groups, two of which were transferred to a diet containing 10 per cent safflower oil. After three weeks, one of the latter groups was returned to coconut oil, and the other was kept on safflower oil for six more weeks. Diet groups were therefore: CN for 12 weeks; CN for 3 weeks, Saff. for 9; and CN for 3 weeks, Saff. for 3, CN for 6. Fatty acids of adipose tissue were extracted and determined by gas-liquid chromatography. Three types of adipose tissue were analyzed: subcutaneous, mesenteric, and interscapular (brown fat). Levels of linoleic acid were higher in the adipose tissue of rats fed coconut oil than in the coconut oil itself. Depletion of adipose-tissue linoleic acid during the first week after transfer from safflower to coconut oil was rapid; the rate then leveled off. Turnover in brown fat was more rapid than in mesenteric or subcutaneous fat. Likewise, during the first week after transfer from coconut oil to safflower oil the increase in the linoleic acid content of adipose tissue was rapid. Later it leveled off somewhat. Percentage of saturated fatty acids increased with time on coconut oil. Interscapular fat had a more constant composition than did mesenteric or subcutaneous fat, and also a higher proportion of nitrogen in relation to fat. The three types of fat were analyzed separately. Half-times of depletion of linoleic acid were calculated, as were the relative percentages of palmitoleic, oleic, lauric, and myristic acids in relation to linoleic acid. Changes in the composition of adipose-tissue fat were toward that of the fats being fed. Equilibration took place more rapidly in females than in males. The half-time of linoleate depletion varied from 11 to 53 days, depending on the age, sex, and kind of adipose tissue. Depletion was more rapid in the interscapular fat. There was a significant drop in total adipose-tissue fatty acid in females between the eleventh and fourteenth weeks of age. When rats were shifted from the linoleate-poor to the linoleate-rich diet, there was some evidence of preferential storage of linoleic acid. Lauric and myristic acids from the coconut oil were stored in smaller proportions and with a quicker turnover than was linoleic acid. Arachidonic acid was found only in trace amounts in adipose tissue. The sharp drop in adipose-tissue fat which occurred in females at the time of maturity was especially noticeable in the saturated-acid fractions.

The liver lipid changes in the same groups of animals differed from adipose tissue changes in the following respects: 1) A higher percentage of phospholipid and cholesteryl ester and a much lower percentage of triglycerides were found in the serum and liver than in adipose tissue. Also, appreciable quantities of free cholesterol, free fatty acids, mono- and diglycerides were found in the plasma and liver. 2) Very little lauric or myristic acid was found in the plasma and liver. In the liver, the arachidonic acid was largely present in the phospholipid, in contrast to the relatively high proportion in plasma cholesteryl ester. Most of the liver cholesterol was esterified with oleic and linoleic acid. 3) Males showed more liver enlargement and total liver storage of cholesteryl ester than did females. Plasma cholesteryl ester arachidonic acid was greater in the females changed from the safflower diet to the coconut oil diet than in similarly treated males. Females seem to store or at least hold on to arachidonic acid to a greater extent than do males. Some plasma cholesterol values were higher in females than in males, and tended to increase with time on diet—that is, with age. 4) Males responded to safflower oil with greater liver enlargement than did females. 5) Triglyceride linoleate in adipose tissue, produced by three weeks of safflower feeding, was still present six weeks after the return from safflower to coconut oil.

The data were evaluated with regard to time of replacement of one fatty acid with another. In the case of linoleic acid in adipose tissue, there seemed to be a rapidly moving and a slower moving fraction. The liver linoleic acid seemed to be found largely in the more rapidly moving fraction. Striking differences between plasma and liver arachidonic acid lay in the fact that in the plasma a large fraction of the arachidonic acid was present as cholesteryl ester arachidonate, while in the liver almost all the arachidonic acid was to be found in the phospholipid. It needs to be noted that all of the animals in this study were autopsied 15 to 18 hours after withdrawal of food cups. Hence, larger proportions of oleic, linoleic, and other fatty acids in plasma and liver might have remained in areas of the less rapidly moving fractions.

Sex Differences in the Metabolism of Lipids

Possible effects of restriction of time of access to food on sex differences in serum cholesterol in rats were investigated at California (*30*). An earlier study (*31*) had shown that estradiol dosage lowered food intake. Pair-fed females showed some tendency to have more consistently high serum cholesterol levels, while males on the same diet showed little effect.

The Lis experiments (*30*) were planned to investigate the validity of three possible hypotheses: 1) that limitation of time of access to food, i.e., pattern of food consumption, might be the primary influencing factor producing sex differences; 2) that a hormone produced in different amounts at different stages of the oestrus cycle might be altering the level of serum cholesterol; and 3) that the high serum cholestrol levels in female rats might be associated with a consistent variation in fatty-acid constituents of cholesterol esters or of other plasma lipids. The latter was considered of particular interest since the increase of serum cholesterol had been found primarily in the esterified rather than in the free cholesterol fraction.

Animals were placed on the usual purified diet, i.e., one furnishing 5 per cent casein plus 10 per cent egg white for protein, 10 per cent fat, and 0.16 per cent choline tartrate. Part of the animals were fed *ad lib.*; part had access to food limited to two hours daily, 8 to 9 a.m., and 4 to 5 p.m. Fats used were coconut (CN) and cottonseed oils (CSO). Reaction of the females to restricted access to food was significantly in the direction of a higher proportion having high serum cholesterol values (fig. 5). In the females fed the cholesterol-rich diet (fig. 5), meal feeding seemed consistently to increase the proportion having high values for plasma cholesterol. Only one group of males fed coconut oil and cholesterol on a restricted regimen had significantly higher plasma cholesterols than did the *ad lib.* animals on the same diet ($p<0.05$) or the males fed no cholesterol ($p<0.01$). The males fed the restricted cottonseed oil-plus-cholesterol diet showed plasma cholesterol values which were not significantly higher than those of the males fed *ad lib.*, but which were higher than those of the males fed cottonseed oil without cholesterol.

Cholesteryl ester fatty acids of males were richer in palmitic than in stearic acid, while values for stearic acid in the cholesteryl ester were higher in females. This was likewise true of the phospholipid fatty acids. Restriction of feeding time lowered the plasma arachidonic acid slightly. However, cholesterol feeding in itself markedly lowered the percentage of arachidonic acid in the plasma cholesteryl ester. This was possibly the effect of dilution, because of the higher percentage of total cholesteryl ester in the plasma. When linoleic acid was available from the diet, it apparently took the place of arachidonic acid in cholesteryl ester. Without it, i.e., when the animals were fed the coconut oil diet, the percentage of oleic acid was increased. The data suggest that the rate of esterification of excess dietary cholesterol may differ in males and females, and that restriction of access to food may delay removal of cholesteryl ester from plasma to a greater extent in females than in males.

An investigation of the liver lipids (*32*) in the same group of animals showed that restriction of time of access to food had little or no significant effect on the total liver lipids and cholesterol in the rats of either sex fed the two diets without cholesterol. When cholesterol was added to the diet, mean values for liver lipids were lower for the restricted males, with both oils, but differences between the restricted and *ad lib.*-fed females on coconut oil were not significant. Indeed, the liver cholesterol values for females on the CN+C restricted diet were somewhat higher than for the corresponding group

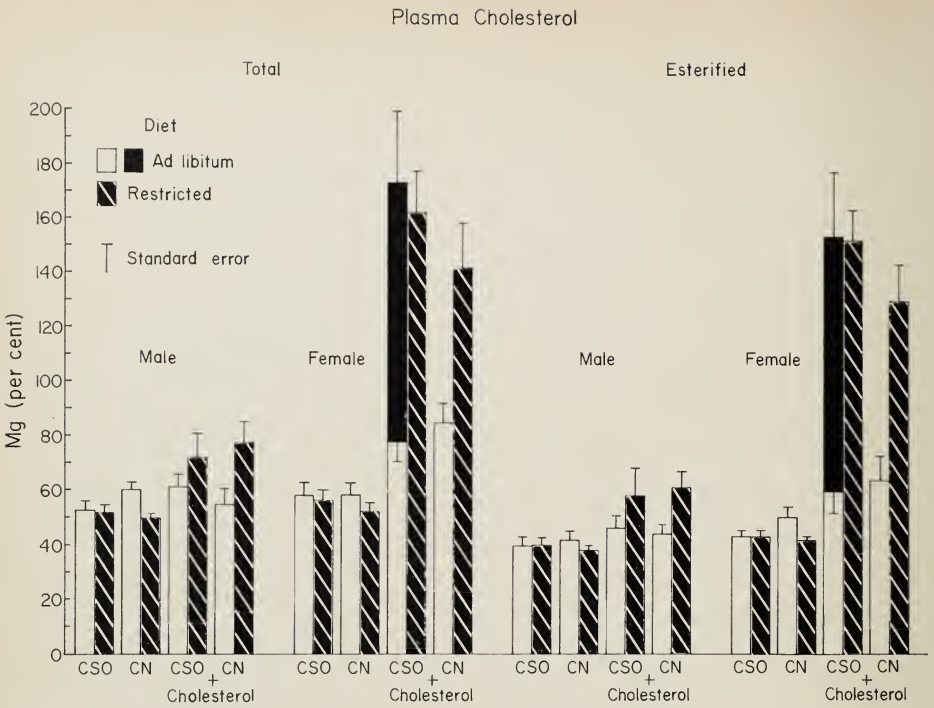

Fig. 5. Total and esterified plasma cholesterol values for rats fed coconut oil and cottonseed oil. Diet groups are indicated as follows: CSO, cottonseed oil only; CN coconut oil only; CSO + cholesterol, cottonseed oil plus cholesterol; CN + cholesterol, coconut oil plus cholesterol; plain columns represent rats fed ad libitum; hatched columns, those with limited access to food. Black areas show proportionate means of high values for females on the CSO diet with cholesterol. (From: Lis, E. W., and R. Okey, Jour. Nutrition **73:** 117 [1961].)

fed *ad lib.* The most interesting data had to do with the distribution of fatty acids in the liver lipid fractions as separated on silicic acid columns and analyzed by gas-liquid chromatography. Arachidonic acid was present chiefly in liver phospholipid, and to a lesser extent in liver cholesteryl ester (CE). The slightly higher percentage of C^{20}:4 in the CE of females was not significant since the total amount was considerably greater in the larger livers of the young males. Linoleic acid was apparently selectively retained in the liver triglyceride (TG) and CE of the CSO-fed rats, while oleic acid tended to take the place of the linoleic acid TG and CE of the rats fed coconut oil. The proportions of stearic and arachidonic acids were somewhat higher in the liver PL and CE of females than of males, but the sex differences were not so great as those for PL and CE of plasma. The proportion of monoenoic and long-chain saturated fatty acid was greater, however, in the CE of liver than of plasma. The authors suggest that there is a tendency toward maintenance of characteristic physical properties rather than of specific fatty acid content of phospholipid and cholesteryl esters of liver and plasma, and discuss the possible function of liver PL fatty acids in esterification of cholesterol.

Another study at California (*33*) reported red-cell and plasma lipids of weanling rats fed 10 per cent fat, with and without 1 per cent cholesterol, for seven weeks. The diets differed from those fed in the preceding study only in that safflower oil (75 per cent linoleate) was used as a linoleate-rich fat instead of cottonseed oil (50 per cent linoleate). Red-cell cholesterol (see note under human red cells) was about three times as high as plasma cholesterol and was practically all unesterified. Sex differences in the plasma phospholipid content of stearic and arachidonic acid were even more apparent than those noted by Lis (*30*). Females also had higher concentrations of plasma cholesteryl arachidonate than did males, but males had consistently higher concentrations of palmitic and linoleic acids in their plasma phospholipids than did females.

The above data with their relatively clean-cut indications of a characteristic sex difference in the fatty acids of phospholipid and of cholesteryl ester led to further investigation of the influence of sex hormones in amounts equivalent to those secreted by intact rats when given to castrates (*34*). The amount of hormone to be administered (estradiol benzoate, 30 μg/wk; testosterone propionate, 2 mg/wk) was established in preliminary experiments, in which growth and liver and adrenal weights of castrated animals with various levels of the hormone were compared with those of intact male or female rats. Estradiol raised the concentration of plasma cholesteryl arachidonate so that it resembled the levels in the female rat. Testosterone-dosed and castrated control rats, however, had cholesteryl arachidonate levels resembling those of the intact male animals. Estradiol also increased the stearic acid: palmitic acid ratio in the plasma phospholipids (table 2), apparently by influencing the amount of stearic and arachidonic acids in the phosphatidylcholines rather than by altering proportions of the individual phospholipid classes. The sex and hormone difference in stearic acid was also evident in the lysolecithin fractions, whereas no sex difference in the arachidonic acid was apparent. This suggested that the arachidonic acid may have been removed, leaving the lysolecithin rich in stearic acid in the females or the estrogen-treated animals. The lysophosphatidylcholine may have originated via the removal and transfer of the arachidonic acid from the β-position of the phosphatidylcholine to the plasma cholesterol. Such a plasma transferase reaction has been demonstrated by others (Glomset *et al.*, *Biochim. Biophys. Acta* **58:** 398 [1962]; *ibid.* **65:** 128 [1962]), but the proportion of cholesteryl ester formed in the plasma by the transferase reaction to that formed by direct esterification in the liver, with subsequent secretion into the plasma, is not as yet known.

For years, it has been recognized that female rats have a lower requirement for essential fatty acid than do males, but the reasons for this sex difference have not been clearly established. As a result of the observation that estradiol alters the metabolism of arachidonic acid in the plasma phospholipids and cholesteryl esters, studies were carried out on intact male and female rats and on gonadal hormone-treated, castrated rats during the development of an essential fatty acid deficiency. An experiment (*35, 36*) was performed in which groups of intact male, female, and estradiol- and testosterone-treated, castrated rats were fed a linoleate-free, but not fat-free (hydrogenated coconut oil) diet for a period of nine weeks. During the development of the deficiency, i.e., at 0, 2, 4, 6, and 9 weeks on the diet, groups of animals were killed, and liver, plasma and total carcass lipids were extracted, fractionated, and analyzed. Results of the study indicated that initially (within two weeks) carcass and organ linoleate was severely depleted, but decreased only very slowly thereafter. Liver phospholipid represented the major source of arachidonic acid in these animals, and even though the males had larger livers, and consequently had more phospholipid, the total liver content of arachidonic acid was the same for both sexes (fig. 6). Eicosatrienoic acid, how-

TABLE 2*

EFFECT OF SEX HORMONES ON CONCENTRATIONS OF PALMITIC, STEARIC, AND ARACHIDONIC ACIDS IN THREE SUBCLASSES OF RAT PLASMA PHOSPHOLIPIDS

Fatty acids of phospholipid fractions†	Per cent of total phospholipid	Concentration in phospholipids of:				
		Intact rats		Castrated rats		
	(av. of group)	Female	Male	Oil control	Estradiol	Testosterone
		mg per cent	*mg per cent*	*mg per cent*	*mg per cent*	*mg per cent*
Cephalin:	5.4					
16:0		0.59	0.56	1.53	0.65	1.51
18:0		0.97	0.90	1.89	0.97	1.61
20:4		1.89	1.59	3.91	1.92	3.29
Stearic/palmitic		1.63	1.60	1.24	1.49	1.07
Lecithin:	57.1					
16:0		7.0	6.9	7.1	7.6	7.0
18:0		9.0	6.1	5.7	8.1	5.3
20:4		20.8	14.7	13.3	18.8	12.7
Stearic/palmitic		1.28	0.87	0.80	1.07	0.75
Lysolecithin:	17.0					
16:0		1.99	2.35	2.11	2.46	2.60
18:0		2.29	1.24	1.23	1.85	1.03
20:4		1.65	1.41	1.45	1.29	1.03
Stearic/palmitic		1.15	0.53	0.58	1.08	0.40

* Table adapted from Lyman *et al.*, *Canadian Jour. Biochem.* **42**: 265 (1964).

† See text for hormone dosage and treatment of animals. Values are averages from analyses of two separate pools of plasma obtained from three or four rats/pool/group. Only three of seven fatty acids analyzed are presented.

ever, was much higher in the males than in the females, indicating that when availability of arachidonic acid is limited, eicosatrienoic acid is substituted, up to a point, in the synthesis of new phospholipid. The results with the castrated, hormone-treated animals were consistent with this interpretation, and it was therefore concluded that an important factor in the relatively greater resistance of the female rat to the effects of an essential fatty acid-deficient diet was due to their slower growth and smaller size. The larger, more rapidly growing male animal is unable to supply arachidonic acid at a rate sufficient to meet its greater needs for phospholipid synthesis; thus it synthesizes and substitutes the abnormal eicosatrienoic acid. Apparently this unnatural isomer is unable to perform the function of arachidonic acid, and deficiency symptoms ensue.

In conjunction with the studies on sex differences in the metabolism of the essential fatty acids, the possibility that estradiol may affect the conversion of linoleic acid to arachidonic acid was also investigated *(37)* in essential fatty acid-deficient, estradiol- and testosterone-treated rats fed linoleic acid 1-^{14}C by stomach tube. After six hours, the relative incorporation and specific activities of linoleic and arachidonic acids in various liver and plasma lipids were determined. The results in table 3 show that most of the label in the liver was concentrated in the phospholipids and triglycerides, whereas it was more evenly distributed in the plasma. Specific activity of plasma cholesteryl ester, however, was

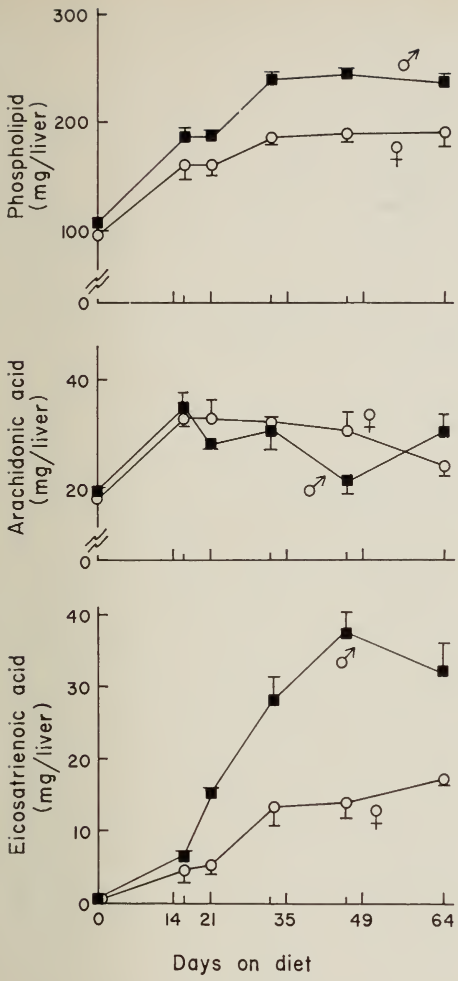

Fig. 6. Liver phospholipid, arachidonic and eicosatrienoic acids from rats during development of an essential fatty acid deficiency. See description of experiment. (From: Ostwald, R., et al., Biochem. Jour. **97:** 485 [1965].)

considerably higher than that of the liver. Plasma cholesteryl linoleate and arachidonate specific activities were also significantly higher than in the liver (table 3), suggesting again that plasma esterification of circulating cholesterol may be important in the maintenance of the fatty acid composition of the plasma cholesteryl esters. There was little evidence, however, that estradiol had any major effect on the synthesis of arachidonic acid from linoleate, although it did appear to cause more labeled arachidonate to concentrate in the liver phosphatidylcholines than did testosterone (data not shown).

Analysis of the plasma lipids of male and female rats during development of an essential fatty acid deficiency revealed that females consistently maintained higher concentrations of arachidonic acid on their plasma phospholipids and cholesteryl esters than did males. While linoleic acid content of these lipids was similar for both sexes, males generally maintained a higher eicosatrienoic acid (fig. 7) (*37*). Thus, in the complete absence of dietary linoleic acid, female rats were able to maintain more normal levels of circulating arachidonate than were males, presumably because of less need for structural purposes. It may be noted that the changes in the unsaturated fatty acids of the plasma phospholipids during the essential fatty acid depletion were reflected by similar changes in the cholesteryl esters. The other fatty acids in these lipids did not follow this pattern. Thus, in the rat, the polyunsaturated fatty acid composition of the plasma phospholipid appears to play an important role in determining the fatty acid composition of the plasma cholesteryl esters.

In other studies (*38, 39, 40*), the California group investigated possible mechanisms by which female rats maintained higher concentrations of stearic and arachidonic acids in their liver and circulating phospholipids. Investigations by others had demonstrated that phosphatidylcholines may be partially separated into groups, depending upon the extent of unsaturation of their fatty acid pairs (Hanahan *et al.*, *Jour. Biol. Chem.* **228:** 685 [1956]; Arvidson, *Jour. Lipid Res.* **6:** 574 [1965]). In addition, those phosphatidylcholine fractions rich in stearic and arachidonic acids apparently incorporated more methyl from $^{14}CH_3$-methionine than did those phosphatidylcholine fractions rich in palmitic and linoleic

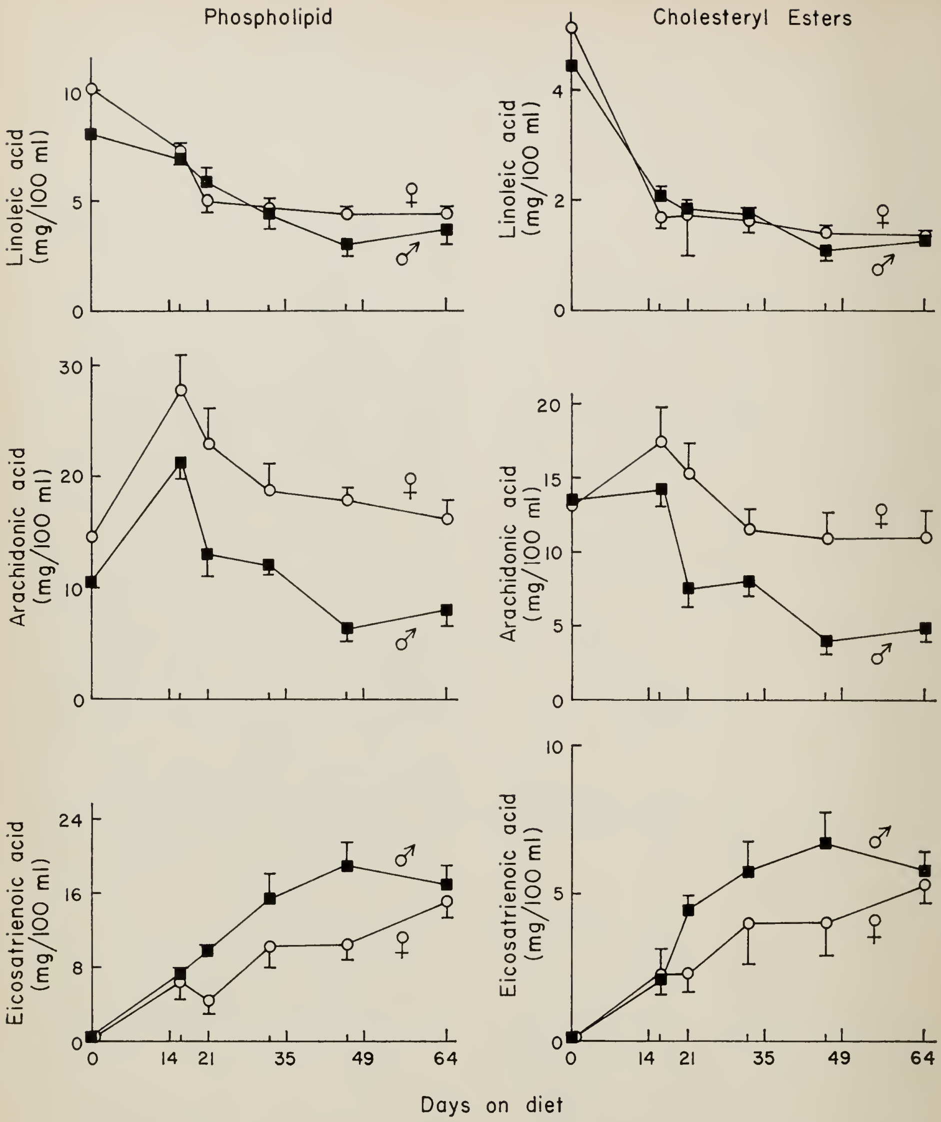

Fig. 7. Concentrations of linoleic acid, arachidonic acid, and eicosatrienoic acid in plasma phospholipids and cholesteryl esters of rats during development of EFA deficiency. (From: Lyman, R. L., et al., Biochem. Jour. **98:** 438 [1966].)

TABLE 3*

SPECIFIC ACTIVITIES OF LIVER AND PLASMA CHOLESTEROL ESTER AND PHOSPHOLIPID†

Lipid fraction and group	Specific activity (cpm/μM FA × 10³)			
	Liver 18:2	Plasma 18:2	Liver 20:4	Plasma 20:4
CE:				
Estradiol	3.73	15.2	0.46	2.17
Testosterone	2.01	11.6	0.35	2.35
PL:				
Estradiol	3.19	3.93	0.66	0.96
Testosterone	3.26	4.40	0.62	2.61

* Table adapted from Ostwald *et al.*, *Jour. Nutrition* **88**: 303 (1966).

† Triglycerides were determined but not shown. Standard errors of the mean have also been omitted.

acids (Isozaki *et al.*, *Med. Jour. Osaka Univ.* **12**: 285 [1962]).

The California experiments (*38*) consisted of injecting male and female rats with methyl-labeled methionine. At various intervals after injection, groups of animals were killed, and the liver phosphatidylcholines were partially separated on silicic acid columns into fractions characterized by different fatty acid content. The proportions of some of the fatty acids in the fractions are shown in table 4. The PC-1 and PC-2 fractions contained the highest proportions of stearic and arachidonic acids, and females had higher proportions of these fatty acids than did males. Incorporation of the labeled methyl to form phosphatidylcholine was maximal one hour after injection and was highest in the PC-1 and PC-2 of the female rats. It was lowest in the fraction having the highest proportion of the palmitic and linoleic acids (fig. 8; table 4). These studies indicate, therefore, that in the rat, female hormone influences the distribution of stearic and arachidonic acids in the liver and plasma phosphatidylcholines by stimulating the metabolic pathway by which phosphatidylcholine is synthesized via methylation of phosphatidylethanolamine. Since the latter phospholipid is rich in stearic, arachidonic and other polyunsaturated fatty acids, more of these acids appear in phosphatidylcholines of the female than of the male. In male rats a greater proportion of phosphatidylcholine seems to be synthesized by the direct pathway in which choline combines with an appropriate diglyceride (Kennedy and Weiss, *Jour. Biol. Chem.*

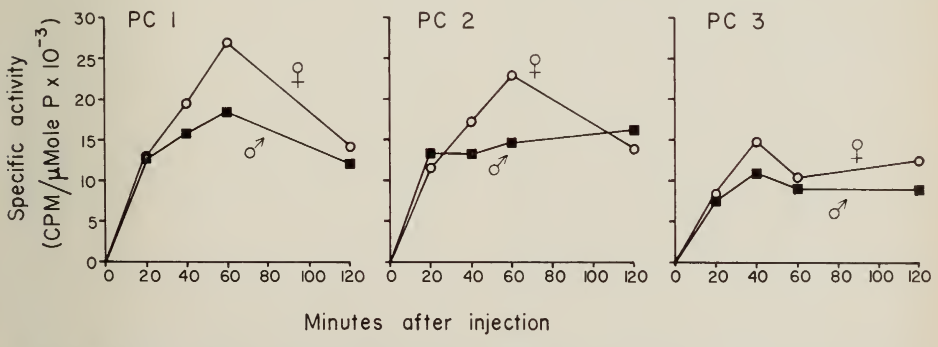

Fig. 8. Specific activities of liver phosphatidyl choline (PC) subfractions at intervals after injection of Me-^{14}C methionine into male and female rats. Each point represents average of four or five rats. (From: Lyman, R. L., et al., Biochim. Biophys. Acta **137:** 107 [1967].)

TABLE 4*

FATTY ACID COMPOSITION OF MALE AND FEMALE RAT LIVER PHOSPHATIDYLCHOLINES†

Compound	Sex	Fatty acids (weight per cent)				Phosphorus (per cent of total recovered)
		16:0	18:0	18:2	20:4	
Phosphatidylcholine-1 (PC1)	M	16.1 ± 1.0	36.1 ± 1.8	4.9 ± 0.7	22.3 ± 2.5	6.6 ± 1.0
	F	10.9 ± 1.6	39.1 ± 2.2	4.5 ± 1.0	27.8 ± 1.2	8.9 ± 1.2
Phosphatidylcholine-2 (PC2)	M	23.3 ± 1.6	30.0 ± 1.8	10.5 ± 1.5	27.5 ± 2.7	12.5 ± 0.9
	F	16.9 ± 0.9	39.9 ± 2.8	5.8 ± 1.1	30.9 ± 2.9	15.0 ± 1.8
Phosphatidylcholine-3 (PC3)	M	28.5 ± 1.2	24.4 ± 1.0	13.6 ± 2.3	13.9 ± 2.9	31.9 ± 2.0
	F	22.3 ± 1.3	31.8 ± 1.4	12.3 ± 1.3	20.5 ± 3.0	28.8 ± 2.6

* Table adapted from Lyman *et al.*, *Biochim. Biophys. Acta* **137**: 107 (1967).

† Values are means ± standard error, of 9 or 10 animals. Phospholipids were separated on TLC, and the phosphatidylcholine spot was divided into three fractions and eluted. The eluants were analyzed for fatty acids and phosphorus. Approximately 46 per cent of the total phosphorus recovered was associated with phosphorus-containing compounds other than phosphatidylcholine, and are not shown.

222: 193 [1956]). The extent to which the sex difference in pathway of lecithin formation may affect properties of lipid-rich tissues remains a fascinating problem. Other aspects of the work covered in papers (*39*) and (*40*) are to be reported as part of the W-91 project now in progress.

Effect of Mineral and Protein Content of Diet

Only one series of studies on the relationships between the mineral content of the diet and lipid metabolism was credited to W-44 R. These were carried out in the Montana laboratory at an altitude of 4,800 feet, and were concerned primarily with relationships between iron metabolism and lipid metabolism. The first paper (*41*) describes rats fed Purina chow for 24 days after weaning (mean weight for males, 186 gm; for females, 145 gm) and then placed on experimental diet. Except for two control diets containing less protein (17.8 and 14.1 per cent), the protein in the experimental diets was furnished by dried skim milk, and represented approximately 24 per cent of the calories. Animal or vegetable fat furnished either 27 or 56 per cent of the calories. Additional minerals from salt mix (USP XIV) were given to two of the groups fed, respectively, animal and vegetable fat at the 56 per cent level. One per cent cholesterol was given to four groups, at two levels of fat intake, one plus and one minus cholesterol. The vegetable fats used were corn oil and Nucoa, but which one was given at any point is not always clear from the publication. Responses to the different fats were much the same as those reported for similarly treated rats from the other laboratories. Serum cholesterols were significantly higher for females than for males, with all the levels of dietary fats, and plus or minus cholesterol. They were higher for both sexes when butter was fed than when either corn oil or Nucoa was fed. Males given the higher levels of fat and cholesterol had higher serum cholesterols than those given the medium level. This was not true for females. Addition of the 2 per cent mineral mix did not alter the significance of the differences in lipid content of tissue and serum, but there was some trend toward correlation of lower serum cholesterols with the higher hemoglobin levels noted when the additional mineral mix was given.

Another paper (*42*) reports investigation of hemoglobin formation and the iron transport system as affected by feeding diets low in protein. Male rats, 8 weeks of age, were fed, for 6 weeks, diets containing 3.5 per cent protein from either beef, ground whole wheat, or ground whole wheat with added lysine. Other rats were fed a chow diet. Adequacy of these diets was measured at the end of the feeding period by the following determinations: hematocrit; hemoglobin; serum iron; total iron-binding capacity of the serum; and serum total cholesterol. An impairment in the iron-transport system and hemopoietic processes was indicated when rats were fed the diets with 3.5 per cent protein from ground whole wheat, with or without added lysine, but not when they were fed a similar diet with 3.5 per cent protein from beef. Rats fed the 3.5 per cent beef- or wheat-protein diets had similar serum cholesterol values. Hematocrit and hemoglobin values and total iron-binding capacity of the serum of the chow-fed rats were somewhat higher than those reported in the literature as standard values for rats, suggesting an increase due to altitude (4,800) ft.). The total serum cholesterol was about the same in rats fed either the beef protein or the wheat protein plus or minus lysine.

A later paper (*43*) reports relationships between biological oxidation mechanisms, serum lipids, and serum-iron transport systems in the rat. Succinic dehydrogenase activities of heart, liver, and kidney of rats on diets known to be hypercholesterolemic were measured. These diets contained USP XIV salt mix with and without the iron removed. All furnished 10 per cent egg albumin, 5 per cent casein, 10 per cent cottonseed oil, and 67.8 per cent sucrose. They were therefore very much like the diets previously reported by California. The experiments were carried out as six replications with 20 rats each. Each replication included two males and two females on each of the following: 1) basal diet; 2) basal diet plus cholesterol; 3) basal diet plus cholesterol plus iron; 4) basal diet plus cholesterol minus iron; and 5) lab chow. Hemoglobin, hematocrits, and serum iron were lower, and serum protein was higher in all rats on the experimental diets than in those on chow. Serum cholesterol was higher in all three of the experimental groups, and serum triglycerides were markedly increased on the experimental diet minus iron. Succinic acid dehydrogenase in heart was not affected by the experimental diet, but it was decreased in liver (on the basis of per gram rat weight) by all three experimental diets. Succinic dehydrogenase content seemed to be more affected by the high cholesterol intake than by low hemoglobin. (*Note:* This is the authors' statement. The editor would add that it might have been related to the lower percentages of functioning tissue in the animals with fatty livers.) Kidney succinic acid dehydrogenase was lower on the experimental diet and especially so in the iron-deficient rats. The authors suggest that the succinic dehydrogenase appeared related to the lower hemoglobin level. When expressed in relation to rat weight, there was more succinic dehydrogenase in the heart and kidney tissues of females than in the similar organs of males. However, the females had slightly less relative succinic dehydrogenase activity in the liver than did the males, when they were fed diets 1, 2, and 4. When fed the iron-deficient diet, females had greater succinic dehydrogenase activity than did males. Although many of the biochemical findings for males and females were significantly different, the direction of response of the two sexes to the dietary variations used in these studies was similar.

Vitamins in Lipid Metabolism

The only published work credited to W-44 and dealing directly with relationships between vitamin intake and lipid metabolism is a series of papers on vitamin A from the University of Idaho. Cholesterol-vitamin A relationships were studied in rats fed different levels of vitamin A (*44, 45*). Weanling male rats were depleted on a diet containing the following percentages of: sucrose, 65.7; vitamin-free casein, 25; cottonseed oil, 5; USP XIV salts, 4; choline, 0.152; the vitamin mix without A, 0.1. Three to five weeks' feeding of the diet to weanling rats produced depletion with the usual signs of deficiency. There were 225 rats in all, approximately 75 in the laboratory at one time. Size of the groups was limited by laboratory space. Vitamin A acetate was fed to each subgroup of 75 at three different levels—10, 150, and 1,000 IU per day. Half of the animals were given 0.5 per cent cholesterol during the period of repletion. Autopsies were carried out after a 24-hour fast for one group and a 12-hour fast for two others. Autopsies and serum and liver analyses were made at 7, 18, and 28 days after the beginning of the repletion period. Totals per subgroup were 14 to 16 rats.

Percentages and total amounts of liver vitamin A increased with time on the repletion diets, whether or not they contained cholesterol (see table 5). When cholesterol was fed for as long as 18 days with 150 or 1,000 IU of vitamin A per day, the total amount of liver cholesterol was lowered. Since 10 IU of A per day exceed normal A requirement of the rat, and the 150 and 1,000 IU per day dosages are so high as to be toxic if continued for any great length of time, there was some question whether the lowering of liver cholesterol storage might have resulted from interference with liver function rather than being a specific effect of vitamin A. Serum cholesterol and serum vitamin A tended to be lowered with increasing time on the 150 and 1,000 IU A repletion diets, whether or not they contained cholesterol. It is possible, therefore, that the animals sacrificed at the 18- and 28-day periods may not have been absorbing the fat-soluble material in the diet to the same extent as the rats in the early stages of repletion. The actual effect of A on cholesterol metabolism is therefore difficult to interpret.

A later report from Idaho (*46*) dealt with the effect of comparatively low dosages of female sex hormone (estradiol benzoate) on cholesterol and vitamin A metabolism. Weanling male rats, half intact and half castrated at 27 to 28 days of age, were depleted of vitamin A as in the previous study (*44*, *45*). Negative controls were autopsied at the end of the depletion period. The remaining 16 groups of 6 animals each were placed on repletion diets for 21 days. Repletion dosages were: 150 and 1,000 IU of A per day, each given to intact or castrate groups—half fed 0.5 per cent cholesterol and half given no added cholesterol. Con-

TABLE 5*

RELATION OF INTAKE TO TOTAL LIVER STORAGE OF VITAMIN A AND CHOLESTEROL DURING VITAMIN A REPLETION OF A-DEFICIENT RATS

Intake		Mean total liver content at:					
		Stage of repletion					
Cholesterol	A per day	7-day		18-day		28-day	
		A	Chol.	A	Chol.	A	Chol.
per cent of diet	*IU*	*µg*	*mg*	*µg*	*mg*	*µg*	*mg*
0....................	10	20	22	27	26	29	26
0....................	150	85	26	196	24	211	28
0....................	1,000	502	26	1,547	26	2,281	29
0.5..................	10	22	137	32	300	25	412
0.5..................	150	93	136	200	226	333	293
0 5..................	1,000	762	148	1,402	226	2,186	264

* Table adapted from Bring *et al.*, *Jour. Nutrition* **85**: 400 (1965).

trol groups were injected subcutaneously with 0.1 ml cottonseed oil (CSO), and experimental groups with 4 μg estradiol benzoate in 0.1 ml CSO, three times weekly.

The author concludes that castrates showed an inverse relationship between liver vitamin A and cholesterol deposition. Estrogen-treated castrates fed cholesterol likewise had lowered liver cholesterol values when given 1,000 IU of A per day. As noted for the previous paper, values may have indicated interference with liver lipid metabolism when these very high dosages of vitamin A were given. Serum of estrogen-dosed animals contained a higher *percentage* of cholesterol than did that of controls, and values for estrogen-dosed castrates were somewhat higher than for intact rats on the same regimen, especially when 1,000 IU of A were given per day. The dosage of estradiol benzoate in this experiment was 12 μg per rat per week as contrasted with the 30 μg given by Lyman *et al.* (*34*). In view of the probable toxic effect of continued high-level dosage of vitamin A, it is difficult to interpret results.

In conclusion, the editor of this review must call attention to the fact that, in spite of the substantial progress made during the years covered by projects W-44 and W-44 R, there are still many unsolved problems in lipid metabolism. Contrasts between findings for human subjects and laboratory animals, and between those for different species of laboratory animals, emphasized the need for study of species differences in lipid metabolism. Laboratories participating in Project W-91, which deals with this subject, are now reporting findings which we hope will contribute much toward solution of the problems of etiology of human atherosclerosis.

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† Findings pertinent to W-91, and therefore not reviewed in detail here.

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